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Directed Antigen Presentation Using Polymeric Microparticulate Carriers Degradable at Lysosomal pH for Controlled Immune Responses

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Abstract: The types of the immune responses generated against an antigen are determined by the intracellular fate of the antigen. Endogenous antigens are processed in the cytoplasm and initiate cytotoxic T lymphocyte (CTL) activation. In contrast, exogenous antigens are degraded in the lysosome (or phagolysosome) of antigen presenting cells (APCs), and induce antibody-mediated immune responses and assist CTL activation. Therefore, maximizing a desired response by controlling delivery pathways is indispensable in vaccine development and immunotherapy. New cleavable microparticles have been prepared for use as protein-based vaccine carriers by polymerizing water soluble monomers including a newly developed aliphatic monomer with a pendant primary amine group and a cleavable acetal linkage with a wholly aliphatic cleavable acetal cross-linker. Incorporation of the cleavable amine monomer in the polymerization mixture increased the encapsulation efficiency of a model antigen, ovalbumin. Ex vivo assays showed that the composition of the particles greatly affected the magnitude and the pathway of antigen presentations, which determine the type of immune responses. The degradable particles synthesized with the new cross-linker enhanced MHC I antigen presentation 2−3-fold over nondegradable particles. It was also found that, by adding 10% cationic cleavable monomers to the microparticles, MHC I restricted antigen presentation was enhanced ca. 75 times over that achieved with nondegradable particles. The microparticles introduced in this study can be further used for targeting and gene delivery due to functionalizable and cleavable cationic monomers in addition to degradability.

Keywords: Microparticle; acetal cross-linker and monomer; CTL activation; antigen presentation; controlled immune response; cancer vaccine

Introduction

For two centuries, vaccines have been used as one of the most efficient therapeutics for preventing and treating many fatal diseases. Currently, there are desperate worldwide needs for vaccines against hepatitis C,[1] AIDS,[2,3] and cancer.[4−6] One of the features distinguishing vaccines from other treatments is that they eliminate their targets such as pathogens and infected cells by activating the immune system rather than

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attacking them directly. To achieve the proper response, the immune system is strictly controlled by complicated networks involving numerous types of cells. Antigen presenting cells (APCs), including macrophages and dendritic cells, initiate immune cascades by presenting fragments (e.g., peptides and lipids) derived from antigens to T cells and by secreting the appropriate cytokines. Antigens from intracellular pathogens such as viruses and bacteria residing in the cell are degraded in the cytosol, and their peptides are presented by major histocompatibility complex class I (MHC I) molecules. Cytotoxic T lymphocyte (CTL) activation is subsequently cascaded from the ligation of a T cell receptor (TCR) on CD8+ T cells and an antigen-derived peptide presented on MHC I by APCs. Finally, the infected cells are lysed by activated CTLs. On the other hand, antigens endocytosed (or phagocytosed) from the extracellular space are processed in acidic lysosomes (or phagolysosomes). The degraded peptides are presented by MHC II molecules and activate CD4+ T cells, which aid in CTL activation and stimulate plasma B cells to produce antibodies.[7] A successful vaccine must be able to activate a desired type of immune response at a sufficient level. For example, it has been shown that a CTL-mediated immune response is more effective than antibody-mediated immunity for eradicating cancer cells.[8−10] However, high levels of antibodies against viruses as well as CTLs against the infected cells are required to prevent and to cure AIDS.[11,12] Therefore, controlling the type of immune response, in addition to achieving sufficient activation, is of great interest in developing a successful vaccine.

So far, targeting phagocytic APCs by delivering antigens encapsulated in the microparticles 0.2−5 μm in diameter has resulted in more efficient immune activation than the direct injection of equivalent amounts of free antigen.[13] However, most of the antigens delivered extracellularly led to MHC II restricted antigen presentation after being processed in lysosome, which is undesirable for cancer vaccine development. However, efficient MHC I restricted antigen presentation is possible if antigens escape from the lysosome into the cytosol rather than being degraded in the lysosome. We have recently validated this hypothesis by showing that enhanced MHC I restricted antigen presentation can be achieved using particles that degrade rapidly at the lysosomal pH of 5.0 leading to disruption of the lysosome.[14,15] Similarly, plasmid DNA encapsulated in degradable particles demonstrated immunostimulatory activity in macrophages, leading to cytokine secretion of IL-6 with a response ca. 40-fold higher than that achieved with DNA alone.[16] Finally, it has been also reported that degradable particles increased the efficiency of a DNA vaccination against cancer.[17]

Here we describe the use of new functional aliphatic monomers and cross-linkers for the preparation of improved microparticles that are degradable in the acidic lysosomes. The use of these building blocks leads to improved properties including increased biocompatibility and faster hydrolysis than observed with our previous building blocks.[14−16] Interestingly, the results of the present study clearly show that the magnitude and pathway of antigen presentation, and thus the types of the immune response, are affected by changes in the level of incorporation and type of cleavable cross-linkers and monomers.

Experimental Section

General. All reagents were purchased from chemical suppliers and used without further purification unless otherwise noted. Acryloyl chloride was distilled prior to use. Tetrahydrofuran (THF) was distilled under nitrogen from Na/benzophenone prior to use. After extractive workup, organic layers were combined and dried with anhydrous $MgSO_4$. To avoid hydrolysis of the acid sensitive compounds during

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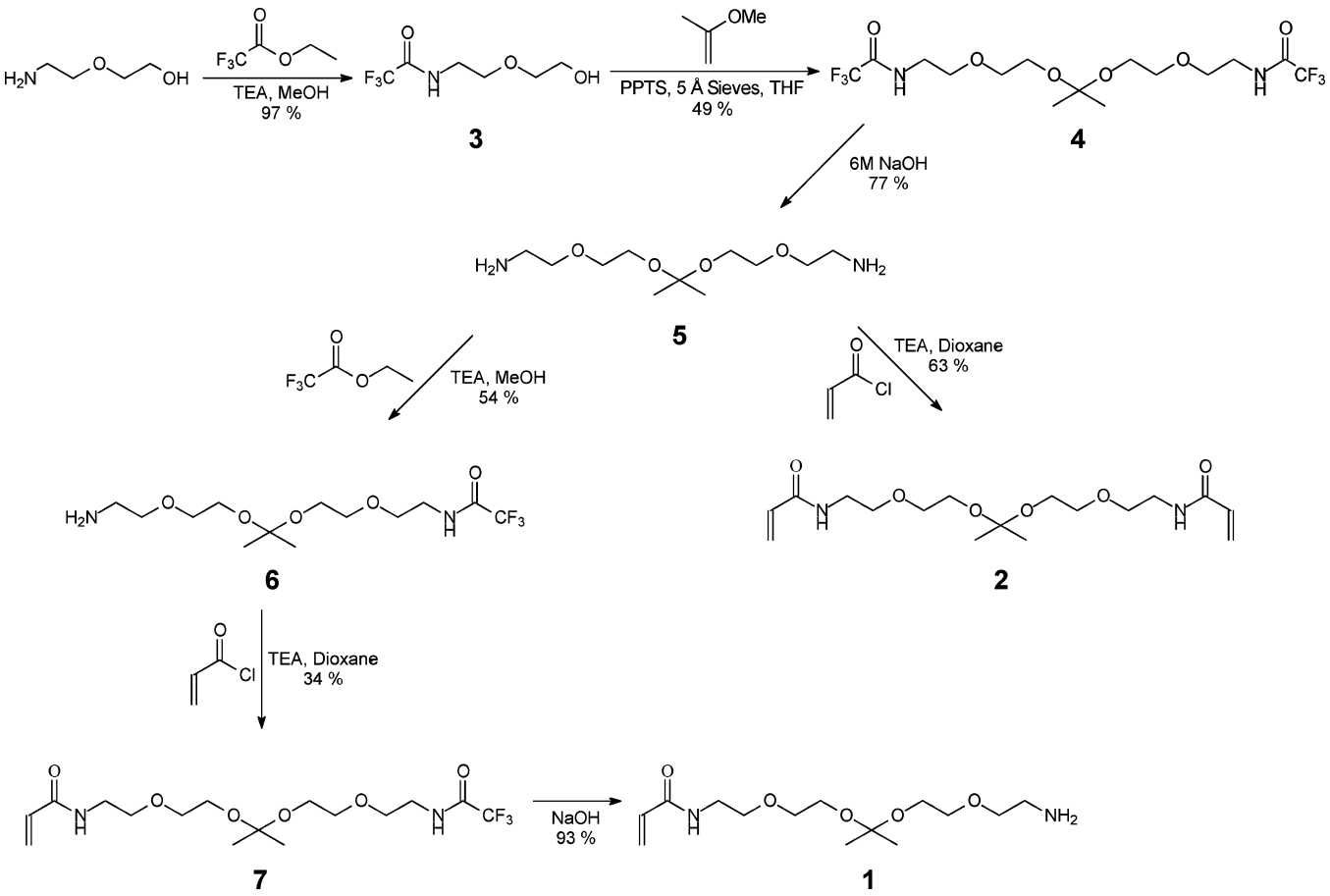

Figure 1. Preparation of the cleavable amine-functionalized monomer **1** and cross-linker **2**.

chromatographic separation on Merck Kieselgel 60 silica gel (230−400 mesh), 1% triethylamine (TEA) was added to the solvents used for elution. For NMR analysis, $CDCl_3$ was filtered through basic alumina prior to use in NMR spectroscopy, which was performed using a Bruker AVB-400 spectrometer. Chemical shifts are given in parts per million relative to tetramethylsilane (TMS), and coupling constants are given in hertz. IR spectra of compounds were acquired as KBr pellets using a Mattson Genesis II FT-IR spectrometer. The overall synthetic scheme is illustrated in Figure 1.

Synthesis of Compound 3. 2-(2-Aminoethoxy)ethanol (15.80 g, 149.46 mmol, 1 equiv) was dissolved in 100 mL of methanol, and triethylamine (24.67 g, 224.08 mmol, 1.5 equiv) was added, followed by ethyl trifluoroacetate (23.34 g, 159.80 mmol, 1.1 equiv). The reaction mixture was stirred overnight at room temperature. Most of the solvent was removed under reduced pressure, then the mixture was dissolved in 50 mL of brine and extracted with 3 × 200 mL of ethyl acetate, and the solvent was evaporated. The crude product was purified by silica gel column chromatography using ethyl acetate as eluent, to afford an amber-colored oil (29.2 g, 145.2 mmol, 97% yield). IR (cm^{-1}): 1133−1206 (br, s), 1711 (s), 3273−3456 (br, s). ^1H NMR (400 MHz, $CDCl_3$): δ 2.79 (s, 1H), 3.52−3.62 (m, 6H), 3.74 (t, J = 4.4, 2H), 7.5 (s, 1H). ^{13}C NMR (400 MHz, $CDCl_3$): δ 39.73, 61.57, 68.82, 72.18, 114.41 (q, J = 287.8), 157.5 (q, J = 37.2). Calcd: $[M + H]^+$ ($C_6H_{11}F_3NO_3$) m/z = 202.0696. Found: FAB-HRMS $[M + H]^+$ m/z = 202.0691. Anal. Calcd ($C_6H_{10}F_3NO_3$): C, 35.83; H, 5.01; N, 6.96. Found: C, 35.54; H, 5.20; N, 7.02.

Synthesis of Compound 4. **3** (11.30 g, 56.18 mmol, 3 equiv) was dissolved in 230 mL of THF, and pyridinium *p*-toluenesulfonate (PPTS) (0.47 g, 1.87 mmol, 0.1 equiv) was added. After 15 min of stirring, 200 g of molecular sieves (5 Å, 1.6 mm pellet) was added and the mixture was stirred for an additional 10 min. 2-Methoxypropene (1.35 g, 18.73 mmol, 1 equiv) was added, and the mixture was stirred overnight at room temperature. After removal of the molecular sieves by filtration, the solvent was evaporated and the mixture of product and reactants was redissolved in 150 mL of phosphate-buffered saline (PBS, pH 7.4). After exhaustive extraction with ethyl acetate, the combined organic layers were evaporated, and the product was purified using silica gel chromatography with 4/1 hexane/ethyl acetate, 1/1 hexane/ethyl acetate, and finally ethyl acetate alone as the eluent. The product (4.07 g, 9.20 mmol, 49% yield) was obtained as white solid. Mp: 46.6−47.8 °C. IR (cm^{-1}): 1185 (s), 1211 (s), 1712 (s), 3306 (br, m). ^1H NMR (400 MHz, $CDCl_3$): δ 1.36 (s, 6H), 3.53−3.63 (m, 16H), 7.03 (s, 2H). ^{13}C NMR (400 MHz, $CDCl_3$): δ 24.71, 39.64, 59.99, 68.52, 70.49, 100.19, 115.86 (q, J = 287.8 Hz), 157.22 (q, J = 37.2 Hz). Calcd: $[M + Li]^+$ ($C_{15}H_{24}F_6N_2O_6Li$) m/z =

449.1698. Found: FAB-HRMS $[M + Li]^+$ $m/z = 449.1698$. Anal. Calcd ($C_{15}H_{24}F_6N_2O_6$): C, 40.73; H, 5.47; N, 6.33. Found: C, 41.05; H, 5.59; N, 6.24.

Synthesis of Compound 5. 4 (3.25 g, 7.35 mmol) was dissolved in 50 mL of 6 M NaOH in water and stirred for 4 h. After extraction with 5 × 100 mL of dichloromethane, the combined organic layers were evaporated, providing a pale yellow oil (1.42 g, 5.67 mmol, 77% yield). IR (cm^{-1}): 1125 (s), 1210 (s), 1573 (s), 3370 (br, s). ^1H NMR (400 MHz, $CDCl_3$): δ 1.31 (s, 6H), 1.63 (s, 4H), 2.79 (t, J = 5.0, 4H), 3.44 (t, J = 5.2 Hz, 4H), 3.53−3.56 (m, 8H). ^{13}C NMR (400 MHz, $CDCl_3$): δ 24.72, 41.59, 59.93, 70.27, 73.09, 99.89. Calcd: $[M + H]^+$ ($C_{11}H_{25}N_2O_4$) m/z = 251.1976. Found: FAB-HRMS $[M + H]^+$ $m/z = 251.1971$. Anal. Calcd ($C_{11}H_{24}N_2O_4$): C, 52.78; H, 10.47; N, 11.19. Found: C, 52.70; H, 10.58; N, 10.93.

Synthesis of Compound 2. Compound **5** (1.07 g, 3.07 mmol, 1 equiv) was dissolved in 10 mL of dioxane with 18.64 g of triethylamine (184.2 mmol, 60 equiv) on ice. Acryloyl chloride (5.56 g, 61.4 mmol, 20 equiv) was added gradually by syringe, followed by the addition of 40 mL of 10% (w/v) potassium carbonate solution and further stirring for 30 min. After extraction with 5 × 25 mL of dichloromethane, the solvent was evaporated. The product was purified by silica gel chromatography using ethyl acetate. The product was obtained as a white solid (0.69 g, 1.93 mmol, 63% yield). Mp: 80.6−81.7 °C. IR (cm^{-1}): 1129 (s), 1549 (s), 1660 (s), 3281 (br, m). ^1H NMR (400 MHz, $CDCl_3$): δ 1.27 (s, 6H), 3.51−3.55 (m, 4H), 3.59−3.62 (m, 12H), 5.64 (dd, J = 10.0, J = 1.6, 2H), 6.12 (dd, J = 16.8, J = 10.4, 2H), 6.30 (dd, J = 17.2 Hz, J = 1.6 Hz, 2H), 6.37 (s, 1H). ^{13}C NMR (400 MHz, $CDCl_3$): δ 24.91, 39.39, 60.00, 69.68, 70.44, 100.07, 126.52, 130.78, 165.59. Calcd: $[M + H]^+$ ($C_{17}H_{31}N_2O_6$) m/z = 359.2184. Found: FAB-HRMS $[M + H]^+$ $m/z = 359.2182$. Anal. Calcd ($C_{17}H_{30}N_2O_6$): C, 56.97; H, 8.44; N, 7.82. Found: C, 56.88; H, 8.43; N, 7.57.

Synthesis of Compound 6. 5 (1.12 g, 4.47 mmol, 1 equiv) was dissolved in 5 mL of methanol with 0.73 g of triethylamine (6.71 mmol, 1.5 equiv), and ethyl trifluoroacetate (0.636 g, 4.474 mmol, 1 equiv) was added. The reaction mixture was stirred at room temperature overnight, and then the mixture was extracted with 5 × 10 mL of dichloromethane. The organic layers were combined and evaporated. The product was purified by silica gel chromatography using a gradient from dichloromethane to 80/20 dichloromethane/methanol to provide the product as a yellow oil (0.84 g, 2.43 mmol, 54% yield). IR (cm^{-1}): 1210 (s), 1561 (m), 1718 (s), 3303 (br, m). ^1H NMR (400 MHz, $CDCl_3$): δ 1.33 (6H), 2.78 (t, J = 5.0, 2H), 3.45−3.52 (m, 4H), 3.55−3.62 (m, 10H). ^{13}C NMR (400 MHz, $CDCl_3$): δ 24.75, 39.83, 41.53, 59.64, 60.17, 68.97, 70.31, 70.40, 72.97, 100.05, 116.05 (q, J = 287.5 Hz), 157.47 (q, J = 37.2 Hz). Calcd: $[M + H]^+$ ($C_{13}H_{26}F_3N_2O_5$) m/z = 347.1795. Found: FAB-HRMS $[M + H]^+$ $m/z = 347.1794$. Anal. Calcd ($C_{13}H_{25}F_3N_2O_5$): C, 45.08; H, 7.28; N, 8.09. Found: C, 45.14; H, 7.45; N, 7.98.

Synthesis of Compound 7. 6 (2.33 g, 6.73 mmol, 1 equiv) was dissolved in 50 mL of dioxane with 40.84 g of triethylamine (403.62 mmol, 60 equiv). The solution was cooled in an ice bath, and acryloyl chloride (12.18 g, 134.54 mmol, 20 equiv) was added slowly via syringe. The reaction mixture was then stirred for 10 min, then 100 mL of 10% (w/v) aqueous potassium carbonate was added, and the reaction mixture was stirred for an additional 10 min. The product was extracted using 3 × 200 mL of ethyl acetate and then purified by silica gel chromatography using hexane, followed by 3/1 hexane/ethyl acetate, 1/1 hexane/ethyl acetate, and ethyl acetate to afford 0.92 g of product as a yellow oil (2.30 mmol, 34% yield). IR (cm^{-1}): 1667 (s), 1716 (s), 3076 (s), 3290 (br, s). ^1H NMR (400 MHz, $CDCl_3$): δ 1.34 (s, 6H), 3.48−3.54 (m, 4H), 3.55−3.64 (m, 12H), 5.62 (dd, J = 10.4 Hz, J = 1.6 Hz, 1H), 6.08 (dd, J = 16.8 Hz, J = 10.4 Hz, 1H), 6.26 (dd, J = 17.2 Hz, J = 1.6 Hz, 1H), 6.31 (s, 1H), 7.36 (s, 1H). ^{13}C NMR (400 MHz, $CDCl_3$): δ 24.80, 39.28, 39.80, 59.97, 60.00, 68.59, 69.56, 70.39, 70.50, 100.14, 115.90 (q, J = 287.8 Hz), 126.53, 130.71, 157.31 (q, J = 37.2 Hz), 165.62. Calcd: $[M + H]^+$ ($C_{16}H_{28}F_3N_2O_6$) m/z = 401.1894. Found: FAB-HRMS $[M + H]^+$ $m/z = 401.1899$. Anal. Calcd ($C_{16}H_{27}F_3N_2O_6$): C, 48.00; H, 6.80; N, 7.00. Found: C, 47.83; H, 7.01; N, 6.75.

Synthesis of Compound 1. 7 (0.5 g, 1.25 mmol) was dissolved in 10 mL of 6 M NaOH in water and the mixture stirred for 30 min. After extraction with 5 × 20 mL of dichloromethane, the combined organic layers were evaporated, to provide the product as a yellow oil (0.35 g, 1.16 mmol, 93% yield). IR (cm^{-1}): 1129 (s), 1549 (s), 1664 (s), 3294 (br, s). ^1H NMR (400 MHz, $CDCl_3$): δ 1.35 (s, 6H), 1.56 (s, 2H), 2.82 (t, J = 5.2 Hz, 2H), 3.47−3.52 (m, 4H), 3.55−3.62 (m, 10H), 5.59 (dd, J = 10.0 Hz, J = 1.2 Hz, 1H), 6.11 (dd, J = 16.8 Hz, J = 10.4 Hz, 1H), 6.23 (dd, J = 17.2 Hz, J = 1.2 Hz, 1H), 6.98 (s, 1H). ^{13}C NMR (400 MHz, $CDCl_3$): δ 24.81, 39.26, 41.79, 59.81, 60.08, 69.82, 70.30, 70.35, 73.43, 99.96, 126.04, 130.86, 165.52. Calcd: $[M + H]^+$ ($C_{14}H_{29}N_2O_5$) m/z = 305.2077. Found: FAB-HRMS $[M + H]^+$ $m/z = 305.2076$. Anal. Calcd ($C_{14}H_{28}N_2O_5$): C, 55.24; H, 9.27; N, 9.20. Found: C, 54.98; H, 9.36; N, 8.89.

Hydrolysis of Cross-linker 2. A stock solution of deuteriated phosphate buffer (D-PBS) was prepared by dissolving 0.03 M KCl, 0.02 M KH_2PO_4, 1.4 M NaCl, and 0.1 M $Na_2HPO_4 \cdot H_2O$ in deuterium oxide. Working D-PBS buffers with pH values of 5.0, 7.4, and 13.0 were then prepared by diluting the stock solution with 10 vol of deuterium oxide and by adjusting the pH with 6 M NaOH. Separate aliquots of the working buffers with pH 5.0 and 7.4 (4 mL) were stirred at 37 °C for 1 min, and the hydrolysis experiments were started by adding 1 mL of the pH 5.0 and 7.4 D-PBS solutions containing 50 mg/mL of cross-linker **2** with rapid stirring. Because some time is required for dissolution and mixing, the time $t = 0$ for this experiment was taken as 30 s after mixing. Aliquots (0.5 mL) of each of the hydrolysis solutions were taken out at various times

Table 1. Formulation and Characteristics of Various Degradable Microparticles[a]

particle	$x/(x+y)$ (%)	$y/(x+y)$ (%)	$n/(x+y+n)$ (%)	diameter (μm) (PDI)[b]	OVA encapsulation (μg/mg)[c]
2	100		5	4.18 (0.168)	57.4 ± 4.8
3	90	10	5	4.12 (0.216)	114.0 ± 8.6

[a] Total amount of monomers and cross-linker was 100 mg in ovalbumin-containing PBS (200 μL). Type 1 particles (5.19 μm in diameter with PDI of 0.187) were made of acrylamide and *N,N′*-methylenebisacrylamide as a nondegradable control. [b] Polydispersity index (PDI) obtained by dynamic light scattering (DLS). [c] Amount of encapsulated protein in nondegradable particle was not able to be determined.

using 1 mL syringes and treated by addition of D-PBS buffer with pH 13.0 (0.2 and 0.6 mL for the samples from pH 5.0 and pH 7.4, respectively) to terminate hydrolysis. The ratio γ of hydrolyzed to total cross-linker at time t was determined by comparing the integrated ^{1}H NMR spectra of the dimethyl peak of the acetal linkage (δ 1.27 ppm) to the vinyl group peak (δ 5.64 ppm). The cross-linker half-life ($t_{1/2}$) was determined as $(\ln 2)/k_d$, where hydrolysis constant k_d was a negative value of the slope obtained by plotting $\ln(\gamma/\gamma_0)$ versus incubation time t. The reference ratio of hydrolyzed to total amount of cross-linker, γ_0, is the ratio at $t = 0$ (30 s after mixing).

Preparation of Microparticles. Microparticles were prepared using an inverse microemulsion polymerization method. Nonionic surfactants (3/1 Span 80/Tween 80, Aldrich, St. Louis, MO) were dissolved in hexane at the concentration of 10% (w/w), and air was removed by sparging with dry nitrogen. An aqueous solution of monomers and cross-linker was prepared by dissolving acrylamide (Biorad, Hercules, CA), cationic cleavable monomer **1**, acetal cross-linker **2**, and 5 mg of ammonium persulfate (APS, Biorad) in 200 μL of phosphate-buffered saline (PBS, pH 8.0) containing 10 mg/mL ovalbumin (Sigma, St. Louis, MO), in the amounts shown in Table 1. The aqueous phase was combined with 20 mL of the organic surfactant solution. After the microemulsion was formed by sonication in a water bath for 30 s, polymerization was initiated by the addition of 20 μL of *N,N,N′,N′*-tetramethylethylenediamine (TEMED, Sigma). The solution was stirred for 10 min before addition of an excess of acetone (1.5−2 times the volume of the organic surfactant solution), followed by centrifugation at 3000 rpm for 3 min. The precipitated pellet was washed once with acetone, centrifuged, and dried under vacuum overnight. For comparison, nondegradable polyacrylamide microparticles were made using acrylamide and nondegradable cross-linker, *N,N′*-methylenebisacrylamide (Biorad).

Measurement of Microparticle Size. Particle sizes were measured using a dynamic light scattering (DLS) particle sizer (Zetasizer Nano ZS, Malvern Instruments, Malvern, U.K.) using a He−Ne laser (633 nm wavelength) with a fixed detector angle of 90°. The microparticles were suspended at 0.1 mg/mL in pH 7.4 PBS (Gibco BRL, Bethesda, MD) at 25 °C and the Z-average size was determined using the software provided by the manufacturer.

Determination of OVA Encapsulation Efficiency. The encapsulated amount of protein was determined using ovalbumin that had been conjugated with the pH-insensitive dye Cascade Blue (Molecular Probe, Eugene, OR). The labeled protein was prepared according to the protocol provided by the manufacturer. Particles were hydrolyzed by incubating with 300 mM acetic acid buffer pH 5.0 for 3 h at room temperature. Ovalbumin concentrations were quantified by measuring the fluorescence of Cascade Blue with excitation at a wavelength of 355 nm and detection at 405 nm using a fluorescence plate reader.

Bone Marrow Derived Dendritic Cells (BMDCs). Femurs and tibia of 2−4 month old female B10.MBR-H2^{bq1}/SxEg mice (Jackson Laboratory, Bar Harbor, ME)[18] were extracted after euthanasia, and the bone marrow was flushed out using a 26 gauge needle with 5 mL of RPMI1640 medium supplemented with 10% (v/v) fetal bovine serum (FBS) (Hyclone, Logan, UT), 100 units/mL penicillin, 100 μg/mL streptomycin, 2 mM L-glutamine, 1 mM sodium pyruvate, and 55 μM 2-mercaptoethanol (Gibco BRL, Bethesda, MD). After the red blood cells were removed by treatment with RBC lysis buffer (Gentra Systems, Minneapolis, MN), the remaining cells were cultured with the medium given earlier and further supplemented with 5% (v/v) of supernatant containing granulocyte-macrophage colony stimulating factor (GM-CSF) kindly provided by Dr. Edward James (UC Berkeley).[19] After cultivation for 7 days in a 6-well plate, the suspended cells were inoculated onto a 96-well plate in the density of 5×10^4 cells/well.

Cytotoxicity Assay. To test the cytotoxicity of the particles, 5×10^4 BMDCs per well of a 96-well plate were incubated with various amounts of microparticles for 6 h. After extensive washing with the medium (3×), the cells were cultured for an additional 36 h. The medium was aspirated, and 10 μL of a stock solution containing 10 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma) in PBS was added along with 90 μL of medium, followed by further incubation for 3 h at 37 °C. The reduced MTT-formazan product generated by live cells was dissolved in 200 μL of dimethyl sulfoxide (DMSO), and 20 μL of glycine buffer (0.1 M glycine, 0.1 M NaCl, pH 10.5) was added. The relative viability of the cells incubated with microparticles to untreated cells was determined by measuring the MTT-formazan absorbance at 561 nm.

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Antigen Presentation Assay. The amount of ovalbumin-derived peptides presented on MHC class I (H-2K^b) and II (I-A^k) molecules was measured by an in vitro cellular assay.[20,21] BMDCs were incubated with varying amounts of ovalbumin-encapsulating microparticles for 6 h and then washed extensively with pH 7.4 PBS. 1×10^5 cells/well of B3Z (CD8+) or KZO (CD4+) T cell hybridomas were co-incubated with BMDCs overnight. These hybridoma express lacZ upon binding of the T cell receptor (TCR) to peptide/MHC. The medium was replaced with 100 μL of CPRG buffer made of 91 mg of chlorophenol red β-D-galacto-pyranoside (CPRG, Roche, Indianapolis, IN), 1.25 mg of NP40 (EMD Sciences, La Jolla, CA), 9 mL of 1 M $MgCl_2$ (Aldrich), and 1 L of PBS. The amount of the lacZ enzyme was quantified by the hydrolysis of CPRG, which produces chlorophenol red absorbing at 595 nm. The quantity of presented peptides was calculated by comparison with the UV absorption values obtained from free MHC I binding peptide, $OVA_{258-265}$ (SIINFEKL), and MHC II binding peptide, $OVA_{247-265}$ (PDEVSGIEQLESIINFEKL) peptides, with B3Z and KZO, respectively, incubated with BMDCs as a reference. The sigmoidal calibration curve was fitted to Gompertz model $Ab = \alpha \exp[-\exp(\beta - \gamma C)]$,[22] where Ab and C are absorbance at 595 nm and particle concentration, respectively. Unknown constants, α, β, and γ, were determined by squared error minimization of experimental measurement with the fitted curve using simulation software MatLab (Mathworks, Natick, MA) under the default conditions. Antigen presentation was determined by extrapolation of experimental data into the generated equation of the calibration curve.

Data Analysis. Unless otherwise indicated, triplicate data were obtained and presented as mean ± standard deviation. Statistical difference was analyzed using analysis of variance (ANOVA) with Student's t test on the significance level of $p < 0.01$.

Results and Discussion

Synthesis of Cleavable Cross-linker and Monomer with Increased Biocompatibility. Typically, exogeneous antigens are processed along the MHC II pathway in lysosomes. However, it has been suggested that disruption of the lysosomal membrane can direct exogeneous antigens into the MHC I pathway.[14,15,23] In previous work we had reported that microparticles showed increased MHC I restricted antigen presentation due to particle degradation in the acidic lysosomal environment leading to membrane disruption. The particles were made using a benzylidene acetal cross-linker that liberated an aromatic aldehyde upon hydrolysis. Since such aldehydes might react with intracellular proteins, we have developed an alternative acid-labile cross-linker **2**, shown in Figure 1, that does not liberate an aldehyde upon hydrolysis. Hydrolysis of novel alkyl acetal **2** produces acetone, a relatively nontoxic metabolic intermediate of fatty acid oxidization. An acid-cleavable alkyl acetal cross-linker with a somewhat similar structure reported previously[24] was not soluble in an aqueous medium. In this study, the new aqueous-soluble cross-linker **2** was synthesized for encapsulation of hydrophilic molecules (e.g., ovalbumin) in the hydrogel particles.

Monomer **1** (Figure 1), used in conjunction with cross-linker **2**, was also designed for its ability to generate small molecules upon acid hydrolysis. This should be beneficial as it was conjectured that the osmotic pressure generated by small molecules released upon hydrolysis of the particles destabilizes the lysosome, thereby contributing to cytosolic release of the encapsulated antigens. In addition to its synergistic action for the formation of small molecules upon hydrolysis, monomer **1** also brings potentially useful additional cationic functionality to the degradable particles, as well as sited for the conjugation of additional biomolecules. While it has been documented that under some conditions primary amines can react with acrylamides by Michael addition,[25] no indications of this reaction were found with acetal monomer **1** and cross-linker **2**.

Hydrolysis of the New Cleavable Cross-linker and Monomer. To test the controlled release of the encapsulated ovalbumin in acidic conditions, acid-cleavable acetal cross-linker **2** was incubated at both physiological and lysosomal pHs (pH 7.4 and pH 5.0, respectively) at 37 °C. Using the Arrhenius equation (i.e., first-order decay), the hydrolysis rate constant and half-lives were calculated (Figure 2). It was found that cross-linker **2** was hydrolyzed approximately 250 times faster at pH 5.0 (half-life of 1.6 min) than at the physiological pH of 7.4 (half-life of 6.8 h). This cross-linker also hydrolyzed approximately 3 times faster than the benzylidene acetal cross-linker used in the previously reported systems.[15] As expected, the structurally related monomer **1** showed almost identical hydrolysis kinetics (data not shown). On the basis of these preliminary results, it was expected that the new acid-labile linkage of **1** and **2** could help control and accelerate release of the encapsulated ovalbumin from the acidic lysosome to the cytoplasm.

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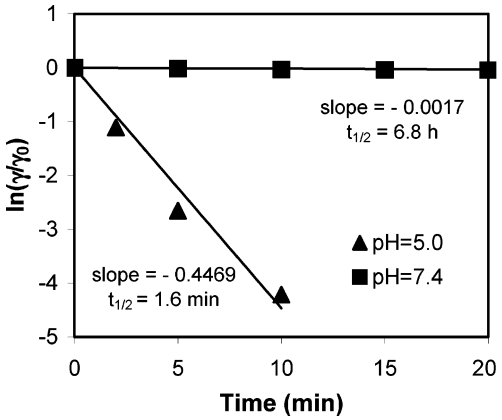

Figure 2. Hydrolysis kinetics of cleavable cross-linker **2** at pH 5.0 and 7.4.

Preparation and Characterization of the Degradable Particles. Three types of microparticles were prepared by inverse microemulsion polymerization of acrylamide using either a noncleavable cross-linker (particle type 1) or a cleavable cross-linker **2** (particle type 2 and 3) (Figure 3 and Table 1). Because cationic particles are internalized by cells more efficiently than neutral ones,[26,27] the cleavable cationic monomer **1** was used to prepare the third type of microparticles (particle type 3). In this instance, the level of incorporation of **1** was set to 10% as previous studies had shown that such a level of positive charge incorporation did not afford unacceptable toxicity.[28] In acidic conditions, the type 2 hydrogel particles are designed to break down into linear polymers and release acetone as well as encapsulated proteins, while type 3 particles would also release amino-ethoxyethanol (Figure 3).

For this study, the size of the microparticles determined by dynamic light scattering (DLS) analysis was of 4−5 μm in diameter (Table 1) for uptake by macrophages and dendritic cells.[17,29] A model antigen, ovalbumin (OVA), was encapsulated in each type of particle. The encapsulation efficiencies of both type 2 and 3 particles were approximately 5−10 times higher than those previously reported,[15] and more OVA was encapsulated in type 3 particles than in type 2 particles under the same conditions (Table 1). This may be due to the attractive interactions between the cationic monomers and OVA, which has an isoelectric point (PI) of 4.6−4.9.[30] It is also possible that the more flexible monomers used in these systems provide more space to accommodate OVA encapsulation.

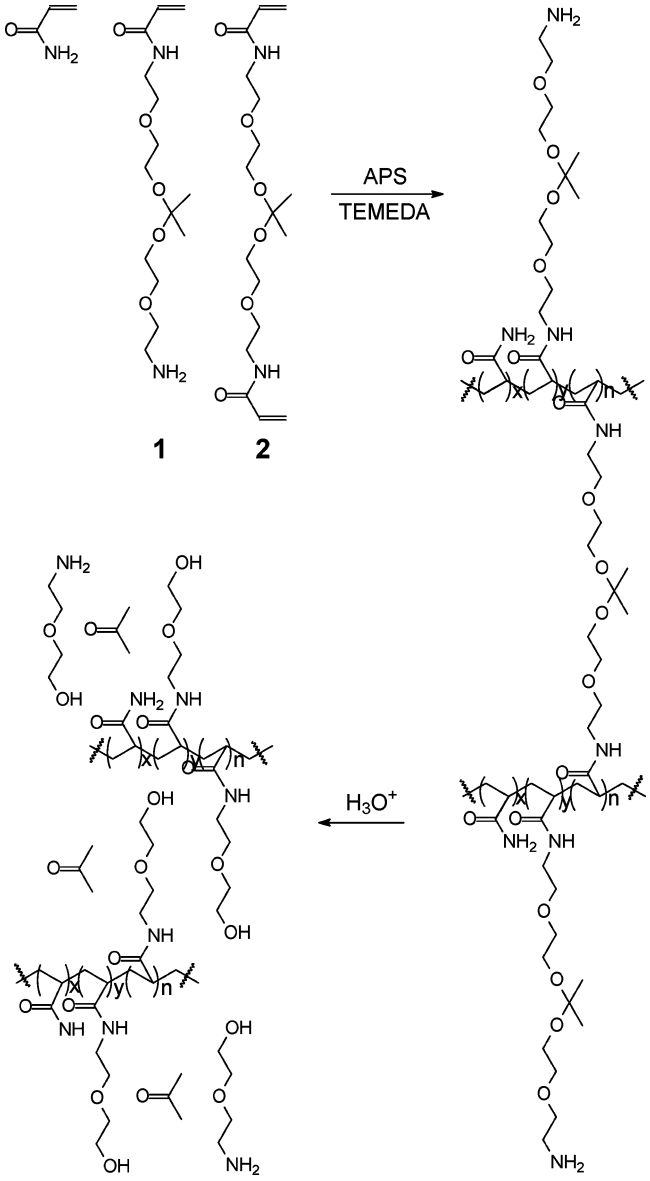

Figure 3. Preparation and hydrolysis of microparticles degradable at lysosomal pH.

Directed and Enhanced MHC I Restricted Antigen Presentations by the New Degradable Microparticles. The performance of the particles as vaccine carriers was evaluated by their incubation with bone marrow derived dendritic cells (BMDCs). DCs are the most potent APCs and play pivotal roles in orchestrating the immune responses.[31−33] Cytotoxicity

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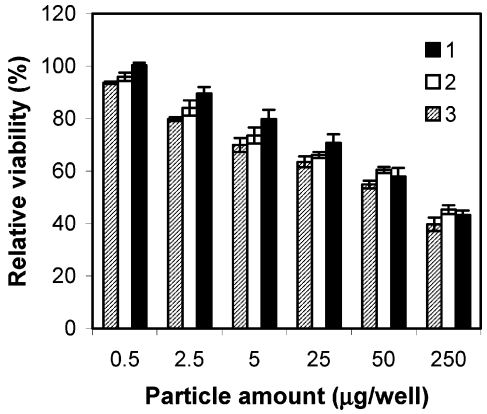

Figure 4. Cytotoxicity of the microparticles at various concentrations after 6 h incubation with BMDCs. Cells were washed extensively after incubation with the particles and further incubated with fresh medium for 36 h. Relative viability was determined by MTT assay.

tests revealed very similar inhibitory effects on the cellular viability and growth with all particles at the concentration range used in this study (Figure 4). The fact that type 2 and 3 particles showed a toxicity similar to that of polyacrylamide particles (type 1), which had been reported as nontoxic to animals, fish, and humans at very high dose,[34] suggests that the type 3 as well as type 2 particles could be administered at high dose in vivo to achieve higher immune responses by targeting more APCs.

Antigen presentation by MHC I and II was quantified by lacZ expression from B3Z (CD8+, Figure 5a) and KZO (CD4+, Figure 5b) T cell hybridomas, respectively, co-incubated with BMDCs, which had been incubated with the microparticles for 6 h. MHC I presentations of antigens delivered by type 2 particles were 1.5−3 times higher than those delivered by type 1 particles at particle concentrations of 25−250 μg/well. Remarkably, antigen presentations by type 3 particles were 75 times higher than those of type 1 particles at the concentration of 25 μg/well (Figure 5a). On the other hand, presentation of OVA-derived peptides by the MHC II pathway using type 1 particles was higher than for type 2 and 3 (Figure 5b). Thus it is clear that the enhanced presentation of OVA-derived peptides by the MHC I pathway rather than the MHC II pathway was achieved by using degradable microparticles (Figure 5c). Type 3 cationic particles were expected to give higher antigen presentation by both MHC I and II than others due to enhanced endocytosis and encapsulation; however, they showed significantly directed MHC class I restricted antigen presentation compared to type 2 particles consisting of only acrylamide and cross-linker **2**. Cross-linker **2** releases only acetone from

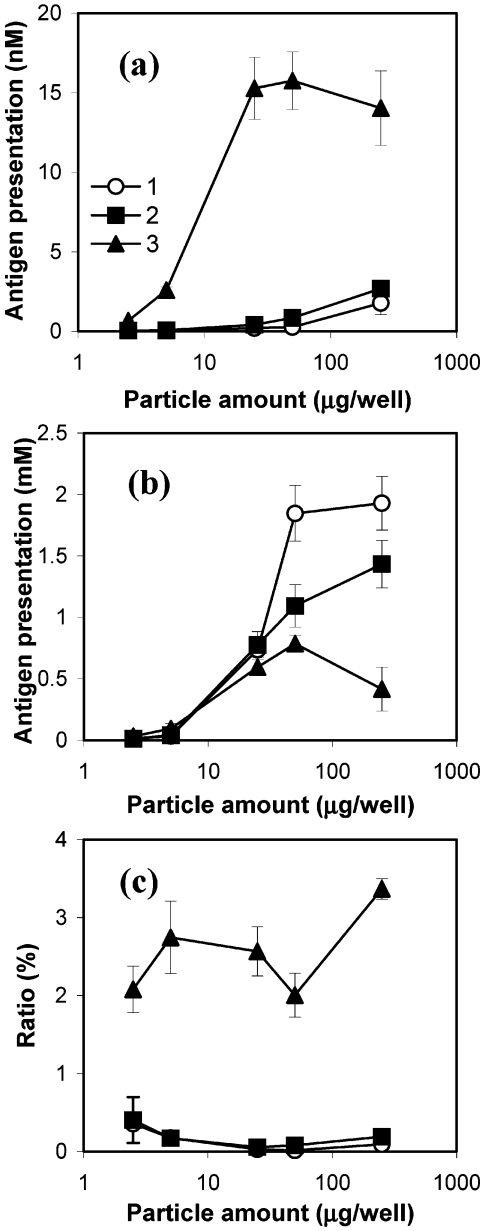

Figure 5. Presentation of OVA-derived peptides restricted (a) by MHC I and (b) by MHC II on BMDCs, and (c) ratio of MHC I to MHC II presentation by BMDCs, after incubation with the particles for 6 h followed by co-incubation with T hybridoma cells overnight.

the polymeric backbones after hydrolysis but monomer **1** releases aminoethoxyethanol in addition to acetone. Therefore, the lysosomal compartment may be more effectively destabilized by type 3 particles than by others due to higher osmotic pressure associated with the release of more small molecules.

Although it is clear that the presence of the cleavable species enhances antigen presentation, optimizing the ratio of monomer **1** and cross-linker **2** is still a challenging task. Monomer hydrolysis releases more small molecules than hydrolysis of the cross-linker, but encapsulated macromol-

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ecules cannot be released until most of the cross-linker moieties are cleaved. A reasonable assumption may be that cleavage of cross-linker **2** determines the rate of antigen release, while degradation of the monomer **1** facilitates the directed MHC I restricted antigen presentation. In addition to the added level of activity they afford, microparticles of type 3 may have yet another advantage. Their incorporation in the polymerization mixture leads to a microparticle with some primary amine groups at the surface; these could be used to conjugate antibodies specific for a target cell or folic acid for cancer targeting. Similarly the presence of amino groups in the interior of the microparticle could be used to advantage to encapsulate oligonucleotides, which would normally easily diffuse out of neutral hydrogel particles, as well as plasmid DNA. Such encapsulation could be achieved at physiological pH due to the cationic interior, while release from the polymeric networks would occur in the lysosome as a result of cleavage of the cationic portion of the monomers. As a result, these features of the particles might be very useful for antisense, immunotherapy, and gene delivery applications.

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